Effects of Methanol Concentration on Expression Levels of Recombinant Protein in Fed-Batch Cultures of *Pichia methanolica*

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Abstract: The methylotrophic yeast *Pichia methanolica* can be used to express recombinant genes at high levels under the control of the methanol-inducible alcohol oxidase (*AUG1*) promoter. Methanol concentrations during the induction phase directly affect cellular growth and protein yield. Various methanol concentrations controlled by an on-line monitoring and control system were investigated in mixed glucose/methanol fed-batch cultures of *P. methanolica* expressing the human transferrin N-lobe protein. The PMAD18 *P. methanolica* strain utilized is a knock-out for the chromosomal *AUG1* gene locus, resulting in a slow methanol utilization phenotype. Maximum growth of 100 g of dry cell weight per liter of culture was observed in cultures grown at 1.0% (v/v) methanol concentration. Maximum recombinant gene expression was observed for cultures controlled at 0.7% (v/v) methanol concentration, resulting in maximum volumetric production of 450 mg of transferrin per liter after 72 h of elapsed fermentation time. © 2003 Wiley Periodicals, Inc. *Biotechnol Bioeng* **81**: 291–298, 2003.

Keywords: *Pichia methanolica* gene expression system; methanol monitoring and control; methylotrophic yeast fermentation; heterologous protein production

INTRODUCTION

The recent application of molecular biology in the production of biopharmaceuticals and proteins of industrial significance has led to the development of heterologous gene expression systems suitable for expression of foreign proteins at high levels. Methylotrophic yeasts are one such system. These yeasts can utilize methanol as their sole carbon and energy source, and have been utilized with great success in biotechnological applications (Egli et al., 1986; Hollenberg et al., 1997; Dale et al., 1999; Cereghino et al., 2000; Gellissen, 2000). The *Pichia* expression systems have a number of desirable features. The yeast host strains are readily amenable to genetic manipulations. Auxotrophic mutant strains facilitate the introduction of protein expression cassettes and the generation of genetically stable expression hosts (Hollenberg et al., 1997; Cereghino et al., 2000). Stability is maintained by the integration of the cassette(s) into the host chromosome (Romanos et al., 1992; Hollenberg et al., 1997; Cereghino et al., 2000). Cultures readily adapt to high-biomass fermentations in a low-cost, defined medium (Cereghino et al., 2000). In addition, these yeasts are able to introduce some eukaryotic-specific post-translational modifications including glycosylation and disulfide bridge formation (Faber et al., 1995; Eckart et al., 1996; Guarna et al., 1997; Hollenberg et al., 1997; Cereghino et al., 2000). The ability to attach a secretion leader such as the *Saccharomyces cerevisiae* α mating factor secretion leader allowing the efficient secretion of properly folded proteins is another advantage of utilizing the *Pichia* expression system. *Pichia* species secrete very low levels of endogenous proteins resulting in easier recovery of an active product directly from the medium (Eckart et al., 1996; Sreekrishna et al., 1997; Cereghino et al., 2000). As a result, methylotrophic yeasts prove to be good host organisms for the production of numerous eukaryotic heterologous proteins (Eckart et al., 1996; Sreekrishna et al., 1997; Cereghino et al., 2000).

Alcohol oxidase mediates the first step of methanol utilization [$CH_3OH + O_2 \rightarrow HCOH + H_2O_2$] (Dale et al., 1999; Cereghino et al., 2000; reviewed by: Koutz et al., 1989; Raschke et al., 1996; Gellissen, 2000). Transcription of the alcohol oxidase genes is completely repressed at moderate levels of glucose or ethanol, but is derepressed by carbon starvation and powerfully induced by addition of methanol

(Guarna et al., 1997; Raymond et al., 1998; Mayer et al., 1999; Cereghino et al., 2000). When induced, alcohol oxidase may comprise up to 35% of total cellular protein (Koutz et al., 1989; Sreekrishna et al., 1997; Dale et al., 1999; Cereghino et al., 2000). The alcohol oxidase promoter has proven to be particularly well suited for driving heterologous gene expression. Adjustment of methanol concentration in the medium controls induction providing tight control of protein expression. This is especially important when producing proteins that inhibit cell growth.

The methylotrophic yeasts *Pichia pastoris* and *Pichia methanolica* share the same methanol utilization pathway (Koutz et al., 1989; Jiménez et al., 1997; Nakagawa et al., 1999). Both species have two alcohol oxidase genes: *AOX1* and *AOX2* in *P. pastoris* (Koutz et al., 1989; Cereghino et al., 2000) and *AUG1* and *AUG2* in *P. methanolica* (Raymond et al., 1998; Nakagawa et al., 1999). In *P. pastoris,* gene expression systems have been developed in which the heterologous gene of interest, under the control of the *AOX1* promoter, is introduced on an integrative plasmid into strains with both functional *AOX* genes (Mut[+] phenotype) as well as strains in which the stronger *AOX1* chromosomal promoter has been disrupted (Mut[s] phenotype). Disruption of both *AOX* genes results in a Mut[-] phenotype (Higgins et al., 1998; Cereghino et al., 2000). The *AOX1* promoter is maximally induced at low methanol concentrations, whereas the *AOX2* promoter is activated at higher methanol concentrations (Nakagawa et al., 1999). Analogous systems have now been developed in *P. methanolica* (Raymond et al., 1998). *AUG1* gene disruptions result in a slow methanol utilization phenotype (Mut[s]); in contrast, a disruption of *AUG2* does not markedly influence methanol-dependent growth, and double-mutant *aug1Δ aug2Δ* (Mut[-]) strains are totally deficient in methanol utilization (Raymond et al., 1998). Although admittedly a contentious issue, some studies have shown that Mut[s] strains may produce higher levels of recombinant proteins than wild type (Mut[+]) strains in certain applications while utilizing substantially less methanol (Cereghino et al., 2000; reviewed by: Faber et al., 1995; Higgins et al., 1998). *P. methanolica* and *P. pastoris* have been shown to produce equivalent levels of recombinant protein when transformed with the similar heterologous protein expression cassettes (Raymond et al., 1998).

In this investigation, we characterized a *P. methanolica* expression system as a function of inducing methanol concentrations that were controlled at constant levels throughout the induction phase of the fermentation by utilizing an on-line monitoring and control system (described by Guarna et al., 1997). We find that tight control of methanol concentrations results in significant improvements in protein yield and cell growth.

MATERIALS AND METHODS

P. methanolica Strain and Inoculum Preparation

The Mut[S] *P. methanolica* strain used in this study, PMAD18, has the genotype [*aug1Δ ade2-11 pep4Δ prb1Δ;*

Raymond et al., 1998]. The *ade2-11* auxotrophic marker facilitates the introduction of *ADE2*-marked expression cassettes by selection for Ade[+]. The strain is also deleted for the *PEP4* and *PRB1* genes that encode the two major vacuolar proteases. The use of protease-deficient mutant strains has been shown to improve the productivity of both intracellular and secreted proteins (Weydemann et al., 1995; Dale et al., 1999; Cereghino et al., 2000). The human transferrin N-lobe protein (hTF/2N) expression strain, PMAD18-p1, was created by transformation of PMAD18 with an expression cassette that harbored the hTF/2N recombinant protein coding region fused to the *AUG1* promoter. Roughly 1,000 Ade[+] transformants were screened by colony Western blots for transferrin expression, and uniform, high-level expression was observed among the majority of expressing strains. Southern blot analysis of several of these strains revealed a single copy of the expression cassette. Standardized cultures were prepared from 10% glycerol frozen stocks. Fermentor starter cultures were prepared by adding 1 mL of glycerol frozen stock to 250 mL of YEPD (yeast extract peptone dextrose media) in a 2-L non-baffled shake flask. Fermentors were inoculated with 60 mL of starter culture grown overnight (18 h) at 30°C.
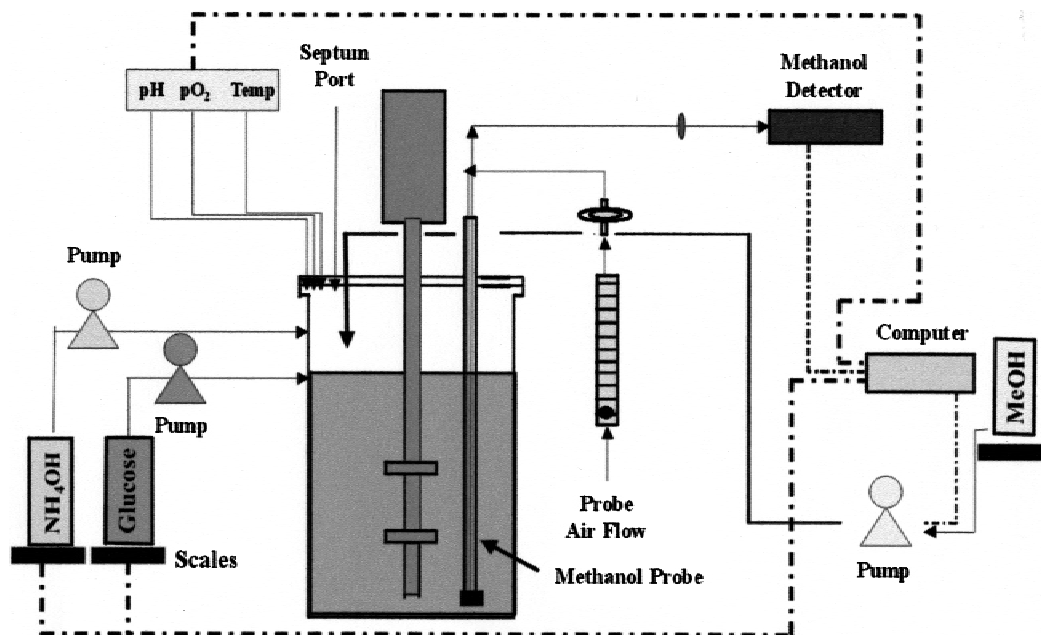
Fermentor Experimental Setup

Fermentations were performed using 3-L fermentors (Applikon Inc., Foster City, CA) equipped with on-line measurement/control of pH, dissolved oxygen, temperature, and methanol concentration (Fig. 1). The fermentors were interfaced with the Applikon ADI 1030 Bio Controller linked to the BioXpert computer based data-acquisition software (Applikon Inc.). Methanol concentrations were monitored and controlled using an on-line methanol sensor/controller (Raven Biotech Inc., Vancouver, BC) interfaced with a methanol feed pump (Masterflex C/L, Cole-Parmer Instrument Co., Vernon Hills, IL). Dissolved oxygen was maintained at 40% air saturation utilizing cascade control of agitation and gas flow/composition. Air sparge was set at 1.0 L/min. Agitation was controlled between a minimum of 375 rpm and a maximum of 1,230 rpm, after which pure oxygen was automatically supplemented to a maximum of 1.5 L/min to maintain 40% dissolved oxygen. Temperature was controlled at 30°C, and pH was controlled at 4.5 utilizing 5 *N* ammonium hydroxide. Nutrient glucose feed and methanol addition were measured using weigh scales (Valley Instrument Corp., Exton, PA) interfaced with the data acquisition software.

Fermentation Conditions

Glucose Batch Phase (10 h)

Media for the initial batch phase of fermentation consisted of 1 L of minimal basal salts medium containing 11.50 g L^{-1} (NH$_4$)$_2$SO$_4$, 2.60 g L^{-1} K$_2$HPO$_4$, 9.50 g L^{-1} KH$_2$PO$_4$,

Figure 1. Schematic representation of 3-L fermentor set-up with on-line monitoring and control of methanol concentration. On-line measurement of pH, dissolved oxygen concentration, temperature, methanol concentration, and nutrient addition are interfaced with a computer data logging system.

and 2.00 g L^{-1} citric acid. Foaming was minimized by adding 1 mL L^{-1} Antifoam 289 (Sigma Chemical Co., St. Louis, MO). After autoclaving and cooling, 48 mL L^{-1} glucose 60% (w/v), 20.0 mL L^{-1} trace elements [containing 100 mM FeSO$_4$ · 7H$_2$O, 2 mM CoCl$_2$ · 6H$_2$O, 1 mM Na$_2$MoO$_4$ · 2H$_2$O, 8 mM H$_3$BO$_3$, 0.5 mM KI, 1 mM NiSO$_4$ · 6H$_2$O, 0.50 g L^{-1} thiamine, and 5.0 mg L^{-1} biotin], 20.0 mL L^{-1} 1 M MgSO$_4$, and 2.0 mL L^{-1} vitamin stock [containing 47.0 g L^{-1} inositol, 23.0 g L^{-1} pantothenic acid, 1.2 g L^{-1} pyrodoxine, 5.0 g L^{-1} thiamine, and 0.1 g L^{-1} biotin] were added.

Glucose Fed-Batch Phase (18–20 h)

Following the 10-h batch phase, continuous glucose feeding commenced. A limited glucose feed profile (based on previous investigations) was adopted to allow for sufficient growth to occur in the Mut^s strain (Fig. 2b). Nutrient feed contained 400 mL 60% w/v glucose, 30 mL trace elements, 30 mL 1 M MgSO$_4$, and 15 mL 50% (NH$_4$)$_2$SO$_4$. Glucose feed rates were programmed to deliver 9.3 g h^{-1} starting at 10 h total elapsed fermentation time, 12.0 g h^{-1} at 18 h, 16.0 g h^{-1} at 30 h, 12.0 g h^{-1} at 40 h, and 9.3 g h^{-1} at 48 h. Cell mass reached 200 g wet cell weight per liter after 28–30 h, at which time methanol addition was initiated.

Methanol Induction Phase after 28–30 h Elapsed Fermentation Time

Based on previous studies, fermentations were induced after 28–30 h, when the cell wet weight reached 200 g L^{-1} of culture. Mixed glucose feed continued following the profile
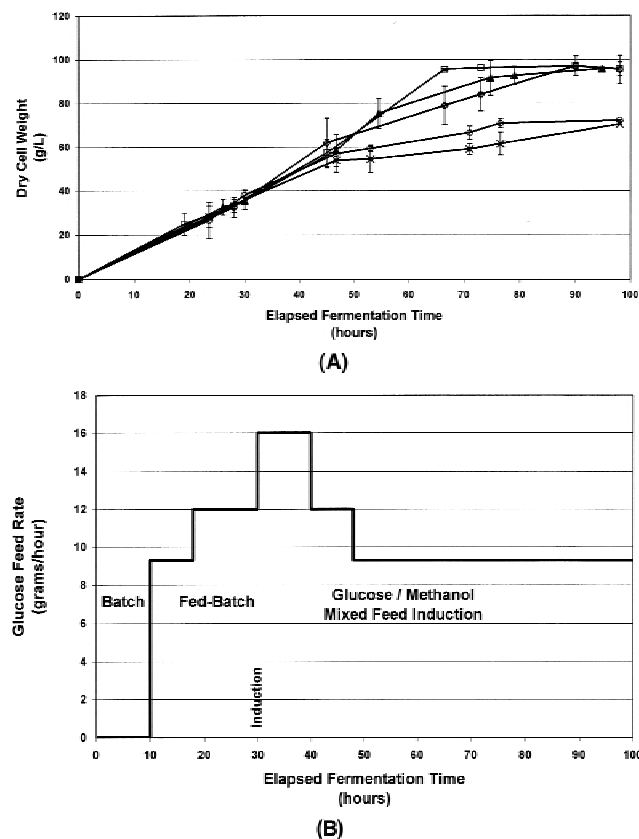
shown previously. Methanol concentration was controlled automatically with 100% methanol delivered in pulses as necessary with a peristaltic pump. The methanol sensor was calibrated at the time of induction by manually adding discreet doses of 100% methanol to the fermentor system. The methanol sensor response has been demonstrated to reach 90% of the steady-state value after 4 min, thus a calibration protocol was established in which stepwise methanol additions of 0.1% or 0.2% increments occurred every 5 min until the desired set point was reached. The sensor is not intrinsically selective for methanol (Guarna et al., 1997), so precaution was taken to avoid vapour interference from any other alcohols or organic solvents, e.g., alcohol was not used to sterilize ports for additions.

Methanol concentrations were controlled between 0.3% and 1.5% in order to establish the effects on both cellular growth and recombinant protein production. Methanol concentrations were controlled at the predetermined level for the entire methanol/glucose mixed feed induction phase. A programmed continuous methanol feed rate of 3 g h^{-1} was also evaluated.

Limitations in fermentor capacity necessitated removal of culture volume, especially for the higher methanol concentrations where methanol volumes added were substantial; this volume was taken into account when calculating total cell mass and transferrin produced. Total fermentation time was 100 h.

Analytical Methods

Samples of the fermentor culture were taken periodically to determine cell density and transferrin production. Cell den-

Figure 2. (A) Effects of methanol concentration on growth in fed-batch cultures of *P. methanolica* Mut^s PMAD18-p1. All cultures (excluding the uninduced controls) were induced at a wet cell weight of 200 g L^{-1} with methanol and subsequently controlled at a constant methanol concentration utilizing on-line methanol monitoring and control system. Cultures were induced with methanol at 28–30 h and controlled at constant methanol concentrations of 0.3% (○), 0.7% (▲), 1.0% (□), and 1.5% (◇). An uninduced culture is also shown (×). (B) Glucose feeding profile utilized for all fermentations. Off-line analysis showed glucose limitation during the methanol/glucose mixed feed induction phase.

sities were measured as dry cell weight (DCW) and wet cell weight (WCW). A 10-mL aliquot of culture was removed from each fermentor and put into pre-weighed conical centrifuge tubes. The tubes were centrifuged at 8,500*g* for 10 min, supernatant samples were removed and frozen at −20°C, and pellets were weighed to obtain the wet cell weight. The pellets were then washed with 10 mL of distilled water, centrifuged, resuspended, and added to pre-weighed aluminum weight dishes. These were placed in a drying oven at 80°C for 48 h and reweighed to obtain the dry cell weight. Supernatant samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) utilizing pre-cast 4–12% Bis-Tris gels (NuPAGE, Invitrogen, Carlsbad, CA), and protein bands were visualized with Coomassie blue stain. Protein bands were quantified by scanning densitometry using purified transferrin as standards.

Chemicals and Reagents

All chemicals used in the preparation of the culture media were obtained from Fisher Scientific (Nepean, ON), BDH Chemical Inc. (Toronto, ON), and Sigma Chemical Company (Oakville, ON). Glucose feed stock was prepared utilizing laboratory-grade 99% pure anhydrous D-glucose from BDH Chemical Inc. All media and solutions were prepared using distilled water.

RESULTS AND DISCUSSION

Methanol Concentration, Cellular Growth, and Recombinant Protein Productivity

PMAD18-p1, a clone of *P. methanolica* expressing recombinant human transferrin N-lobe protein (hTF/2N), was utilized in this investigation. The host strain, PMAD18, is a knock-out for the chromosomal *AUG1* locus (*aug1Δ*), producing the Mut^s phenotype, and an adenine biosynthesis auxotroph (*ade2-11*), allowing selection of clones that have taken up an *ADE2* expression cassette (Raymond et al., 1998). Because of the deficiency in methanol utilization, a mixed nutrient feed must be supplied to provide an alternate carbon source in the form of glucose to promote growth of the organism throughout the induction phase.

The PMAD18 strain also has engineered deletions in the *PEP4* and *PRB1* genes [*pep4Δ prb1Δ*] that are responsible for the majority of protease activity in yeast (Sreekrishna et al., 1997; Higgins et al., 1998; Raymond et al., 1998; Cereghino et al., 2000). Although *Pichia* and *Hansenula* demonstrate limited proteolysis compared to classical systems, such as *S. cerevisiae* (Weydemann et al., 1995), the use of protease-deficient mutant strains has been shown to improve the productivity of both intracellular and secreted proteins (Weydemann et al., 1995; Dale et al., 1999; Cereghino et al., 2000).

In traditional *Pichia* fermentation protocols, methanol concentration is estimated indirectly by interrupting the methanol feed and measuring the time required for methanol to be exhausted as indicated by a sharp increase in dissolved oxygen (i.e., "DO spike"). This approach, however, repeatedly exposes the cells to potentially non-inducing levels of methanol and is restricted to clones having the wild type phenotype for methanol utilization (Mut$^+$). Clones with the disrupted *AUG1* gene, exhibiting the slow methanol utilization phenotype, Mut^s, consume methanol at a rate that is too slow for this technique to be practical. An on-line methanol monitoring and control system allows accurate control of methanol avoiding the periods of methanol exhaustion and build-up. Methanol concentration is a critical parameter in the cultivation of *Pichia* since the level of methanol affects both growth and the expression of the heterologous gene. The optimum methanol concentration to maximize heterologous protein production is not known. It likely varies depending on the methanol utilization phenotype. Mut$^+$ clones are routinely grown under limited methanol conditions. A previous report has shown that, for a Mut^s strain of *P. pastoris,* control at a methanol concentration of 0.3% (v/v) methanol increased recombinant protein produc-

tivity compared to traditional interrupted fed-batch inductions utilizing a maximum of 0.5% methanol suggested in the *Pichia* Expression Kit (Invitrogen, Carlsbad, CA) (Guarna et al., 1997).

Fed batch fermentations of PMAD18-p1, a Mut[s] clone of *P. methanolica,* were grown at different controlled methanol concentrations to optimize recombinant protein production. Figure 2 shows the effect of methanol concentration on cell growth. The glucose feed profile used in these experiments (shown in Fig. 2b) was based on previous studies by ZymoGenetics (unreported results). The objectives of this feeding strategy were to maintain limiting glucose concentration during the induction phase, since glucose is a powerful repressor of the *AOX1* promoter (Raymond et al., 1998), but to support growth to high cell density (approximately 100 g L^{−1}) in mixed feed cultures with methanol. Higher glucose feed rates may have increased the growth rate of this Mut[s] strain, but would have increased the danger of repression either by residual glucose or by the production of ethanol by the Crabtree Effect (Flores et al., 2000). Offline measurements indicated negligible levels of residual glucose in the medium (results not shown). The possibility of ethanol accumulation is a relevant concern particularly because the methanol sensor cannot distinguish between methanol and ethanol. *P. pastoris,* however, has been shown to be a poor fermentor, having a strong preference for respiratory growth, decreasing the possibility of ethanol build up in high cell density cultures that may be seen in more traditional yeast systems (Cereghino et al., 2000). Offline assays for ethanol were not performed; however, it seems unlikely that ethanol was produced with this glucose feed profile since there was no evidence of alcohol (or other flammable vapors) in the methanol sensor traces from the uninduced cultures.
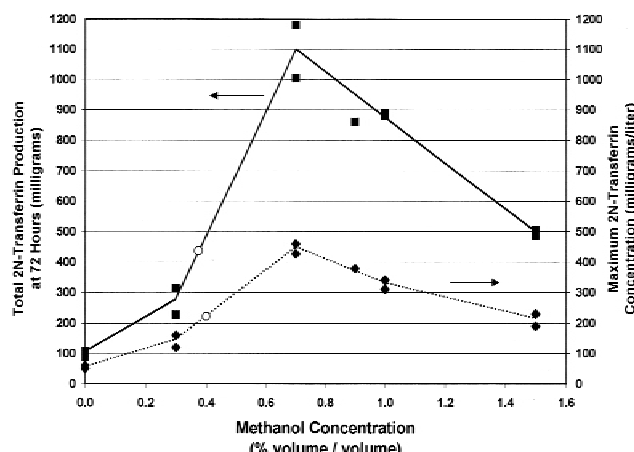
The glucose feed profiles (Fig. 2b) were the same for all fermentations, so any differences in cellular growth are assumed to be a result of methanol metabolism. Alcohol oxidase is derived only from the *AUG2* gene since *AUG1* is disrupted. Cultures were run for up to 100 h, but maximum transferrin concentrations were always attained by 72 h of total culture time. For comparison purposes we used this first 72-h period. At a methanol concentration of 0.3% (v/v) only 72 g dry cell mass per liter was reached after 72 h, slightly more than the uninduced culture dry cell weight of 60 g per liter. Higher methanol concentrations result in a corresponding increase in cellular growth. Maximum growth occurred at an induction concentration of 1.0% methanol, with a maximum dry cell mass of almost 100 g per liter. These data are consistent with the interpretation that the *AUG2* promoter is only weakly induced at low methanol concentrations and that *AUG2*-encoded alcohol oxidase can support limited methanol-dependent growth. Methanol concentrations above 1.0% support little additional cellular growth.

In Mut[+] strains of *P. pastoris,* toxic effects of high methanol concentration are observed, and this is presumed to be due to the buildup of formaldehyde and H_2O_2. Methanol

utilization in the *P. pastoris* and *P. methanolica* Mut[s] expression systems are limited compared to a wild-type strain, even at high methanol levels, presumably reducing the accumulation of formaldehyde.

Recombinant protein expression under the control of the strong *AUG1* promoter for different methanol concentrations is displayed in Fig. 3 and summarized in Table 1. Samples taken prior to induction showed little or no recombinant hTF/2N protein production. Glucose becomes limiting after approximately 28 h at a wet cell weight of 180–200g L^{−1}, previously verified by off-line glucose analysis (data not shown). After this time small amounts of hTF/2N are produced in the absence of methanol induction, with uninduced cultures demonstrating an average maximum transferrin concentration of 55 mg L^{−1} of hTF/2N corresponding to a total production of 98 mg of hTF/2N at 72 h (based on the total volume of the culture at this time).

Fermentations controlled at 0.3% (v/v) methanol showed a 3-fold increase in volumetric protein productivity compared to the uninduced culture, producing a final concentration of hTF/2N of approximately 140 mg L^{−1}, and a total production of 270 mg of hTF/2N (at 72 h). Maximum hTF/2N production was seen at a methanol concentration of 0.7%, with a maximum hTF/2N concentration of 450 mg L^{−1}. The total transferrin production was 1,100 mg (at 72 h), more than 10-fold higher than the uninduced cultures and 4-fold higher than cultures controlled at 0.3% methanol. Controlling the methanol concentration at 1.0% resulted in decreases in both the volumetric and total recombinant protein production. Fermentations controlled at 0.7% and 1.0% methanol utilized more methanol than the 0.3% methanol culture; therefore final culture volumes were substantially higher, resulting in an even greater increase in the total hTF/2N production based on a 72-h harvest. For all of the induced cultures, the maximum transferrin concentration,

Figure 3. Effects of methanol concentration on 2N-transferrin production in fed-batch cultures of *P. methanolica* Mut[s] PMAD18-p1. Maximum transferrin concentration (mg L^{−1}) for each controlled methanol concentration is shown (♦). Total 2N-transferrin (mg) produced at 72 h is also shown (■). Culture conditions are as in Fig. 2. A continuous methanol feeding strategy of 3 g h^{−1} is also shown (○). Note that culture volumes increased with increasing methanol addition.

Table I. Summary of results for mixed glucose/methanol fed-batch cultures of *P. methanolica* Mut[s] PMAD18-p1; results show average values determined from replicate experiments.
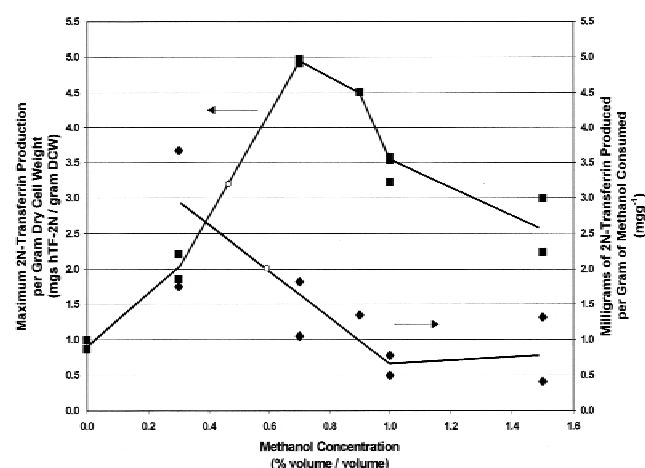
Methanol concentration (%)	Maximum DCW (g/L)	Maximum 2N-transferrin concentration (mg/L)	Culture volume at 72 h (mL)	2N-Transferrin produced at 72 h (mg)	Total methanol utilized at 72 h (mL)
Uninduced	60.6 ± 5.3	55 ± 7	1,779 ± 28	98 ± 14	0
0.3	72.4 ± 0.4	140 ± 28	1,921 ± 43	270 ± 61	80 ± 34
0.7	92.6 ± 4.3	444 ± 23	2,457 ± 153	1,092 ± 125	449 ± 120
1.0	96.9 ± 1.4	325 ± 21	2,723 ± 153	884 ± 8	702 ± 144
1.5	96.9 ± 4.5	210 ± 28	2,385 ± 387	496 ± 14	466 ± 180
Programmed continuous feed (3 g h⁻¹)	69.9 ± 5.8	217 ± 9	2,024 ± 51	438 ± 30	163 ± 2.5

and hence the maximum volumetric protein production, was seen at 72 h.
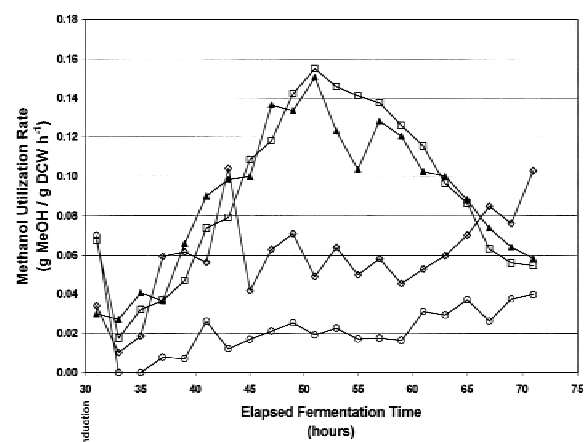
It is interesting to note the differences in methanol concentration for maximal growth and transferrin production. Our data also suggests that recombinant protein production is more sensitive to methanol induction levels than cell growth. A sharp drop in volumetric protein productivity is seen above 0.7% methanol, whereas growth is not inhibited by methanol levels up to 1.5% (Fig. 3). Figure 4 shows the amount of recombinant protein produced per gram of methanol consumed. It is evident that at lower methanol concentrations, the effect of methanol is to drive the expression of the recombinant protein cassette. As methanol levels increase, more methanol is utilized for growth while still maintaining strong induction of recombinant protein expression. Cell productivity was very sensitive to methanol concentration with a 5-fold increase in transferrin produced per gram cell dry weight when the inducer concentration was

raised to the optimum level of 0.7% and a rapid decrease in production at higher levels (Fig. 4). It is apparent that methanol concentration drives both an increase in the number of cells in the culture media and an increase in the cellular productivity which contribute to the overall increase in both volumetric and total protein production.
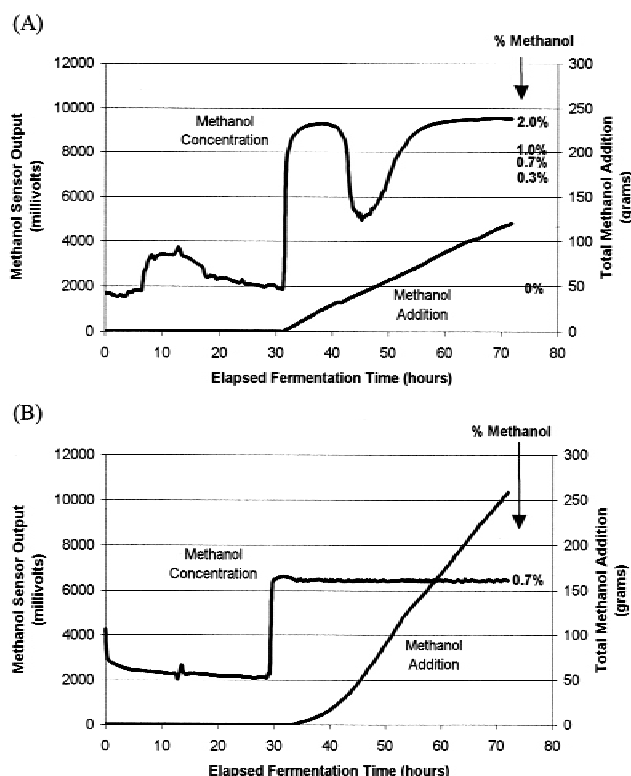
Figure 5 shows the methanol utilization rates over the course of the 72-h fermentation for the various methanol set points. A lag period of 3–4 h is observed for all methanol concentrations as the cells adjust the metabolism from limited glucose feed to methanol utilization. A similar transition period of 2 h was demonstrated by Rodriguez Jiménez et al. (1997) in *P. pastoris* cultures induced with 1.0% methanol. Methanol utilization rates peak at 0.15 g g⁻¹ dry cell weight per hour after 21 h of induction for controlled methanol induction concentrations of 0.7% and 1.0%, suggesting that the highest rate of protein production may occur at this time. Methanol concentrations controlled at 0.3% limit the induction potential of the *AUG* genes as can be seen by the diminished methanol utilization rates. The toxic

Figure 4. (◆) Total 2N-transferrin produced per gram of methanol consumed in fed-batch cultures of *P. methanolica* Mut[s] PMAD18-p1 based on a 72-h harvest time. Points represent separate experiments. (■) Maximum 2N-transferrin produced per gram dry cell weight in fed-batch cultures of *P. methanolica* Mut[s] PMAD18-p1. Culture conditions are as in Fig. 2. Cultures were induced with methanol at 28–30 h and subsequently controlled at a constant concentration. A continuous methanol feeding strategy of 3 g h⁻¹ is also shown (○).

Figure 5. Influence of methanol concentration on methanol utilization rates in fed-batch cultures of *P. methanolica* Mut[s] PMAD18-p1. Methanol utilization rates are expressed in grams of methanol added per gram of dry cell weight per hour. Cultures were induced with methanol at 28–30 h and subsequently controlled at constant methanol concentrations of 0.3% (○), 0.7% (▲), 1.0% (□), and 1.5% (◇).

Figure 6. Methanol concentration and methanol addition versus time in fed-batch cultures of *P. methanolica* Mut^s PMAD18-p1. (**A**) Culture induced at 28 h utilizing a programmed continuous methanol feeding strategy of 3 g h^{-1}. (**B**) Culture induced with methanol at 28 h and subsequently controlled at a constant methanol concentration of 0.7% (v/v) utilizing an on-line methanol monitoring and control system. The methanol sensor on-line calibration results are shown at right.

effects of a methanol concentration of 1.5% are evident as utilization rates decrease significantly at this point.

Industrial *Pichia* fermentations frequently use a programmed methanol feed rather than a feedback control system to regulate methanol concentration in the culture medium. For comparison we examined the methanol addition and concentration as a function of time in a culture receiving a programmed continuous methanol feed of 3 g h^{-1} and a culture controlled at 0.7% methanol (Fig. 6a and b, respectively). Tight control of methanol using the on-line methanol sensor results in a constant level of inducer throughout the culture. With the programmed feed culture, methanol rapidly accumulated to more than 2.0% during the transition phase to methanol metabolism. This was followed by depletion of methanol as the cell densities increased. The methanol concentration accumulated again after 41 h as the cells entered stationary phase. It is not clear how these erratic changes in methanol concentration affect the physiology of the yeast; however, it is clear from Figs. 2 and 3 that both cell growth and transferrin production were suboptimal.

It should be noted that the PMAD18-p1 clone used in this study carried only one copy of the hTF/2N gene. This was intentional so that a series of single-copy *P. methanolica*

clones differing in methanol utilization and protease phenotypes could be compared. This work is in progress. In the context of the current study, the absolute values of the maximum transferrin production either on a volumetric or a total culture volume basis could probably be increased substantially by selecting a multi-copy clone.

CONCLUSION

On-line monitoring and control of methanol concentrations in mixed glucose/methanol fed-batch cultures of a Mut^s clone of the methylotrophic yeast *P. methanolica* has proven effective in substantially increasing the yield of recombinant transferrin and increasing cellular growth. Tight control of methanol inducer concentration leads to an increase in recombinant protein productivity by avoiding the periods of buildup of methanol to cytotoxic levels and depletion of methanol to non-inducing levels. The increased methanol addition rate for programmed continuous feeding resulted in maximum growth yields similar to the 0.3% controlled culture even though greater volumes of methanol were fed, demonstrating the negative effect of the methanol fluctuations. Interestingly, we observed that optimal concentration of methanol for protein production differed significantly from the optimal concentration for cell growth. Overall, these studies demonstrate that tight control of methanol induction levels and empirical determination of product levels are critical for obtaining optimal yields of recombinant protein from fermentations of recombinant methylotrophic yeast systems.

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